Superoxide Contributes to Development of Salt Sensitivity and Hypertension Induced by Nitric Oxide Deficiency

Libor Kopkan, Dewan S.A. Majid

Abstract—This study was performed to examine the role of superoxide ($O_2^-$) in the development of salt sensitivity and hypertension by inhibition of nitric oxide (NO) generation. Male Sprague-Dawley rats were fed with diet containing either normal salt (NS) (0.4% NaCl) or high salt (HS) (4% NaCl). These rats were treated with or without an NO synthase inhibitor, nitro-L-arginine methylester (L-NAME) (15 mg/kg/d) and O$_2^-$ scavenger, tempol (30 mg/kg per day) in the drinking water for 4 weeks. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography and urine collection was performed during the course of experimental periods. At the end of 4 weeks, L-NAME treatment resulted in greater increases in SBP in HS rats (127±2 to 172±3 mm Hg; n=8) than in NS rats (130±2 to 156±2 mm Hg; n=9). Co-administration of tempol with L-NAME markedly attenuated these SBP responses to a similar level in both HS (128±3 to 147±2 mm Hg; n=8) and NS rats (126±2 to 142±3 mm Hg; n=8). Urinary 8-isoprostane excretion ($U_{isoV}$) increased in response to L-NAME treatment that was higher in HS (10.6±0.5 to 21.5±0.8 ng/d) than in NS rats (10.8±0.7 to 16.9±0.6 ng/d). Co-treatment with tempol completely abolished these $U_{isoV}$ responses to L-NAME in both HS and NS rats but did not alter urinary $H_2O_2$ excretion rate. The decreases in urinary nitrate/nitrite excretion in response to L-NAME treatment were not altered by co-administration of tempol in both HS and NS rats. These data suggest that enhancement of $O_2^-$ activity during NO inhibition contributes to the development of salt sensitivity that is associated with NO-deficient hypertension. (Hypertension. 2005;46[part 2]:1026-1031.)

Key Words: hypertension ■ kidney ■ nitric oxide

Superoxide ($O_2^-$) and other reactive oxygen species are constant products of cellular metabolism. However, the development of oxidative stress is dependent on the balance between their production and degradation.1,2 $O_2^-$ is usually instantly reduced by the enzyme superoxide dismutase (SOD) normally present in living tissues.3 Nitric oxide (NO), another free radical, is also known to act as an antioxidative agent by constant elimination of $O_2^-$ from the tissue, thus helping to maintain minimal level of $O_2^-$ in normal condition and provides a protective function against the action of $O_2^-$ in many organs including the kidney.4–6 Thus, it is possible that in the condition of NO deficiency, there is an increasing accumulation of $O_2^-$ in biological tissues.5 In fact, we have observed in an earlier study that acute NOS inhibition leads to enhanced $O_2^-$ activity that exerts vasoconstriction, antiure- sis, and antinatriuresis effects in the kidney.6,7 The results of these studies indicate that NO deficiency can lead to the development of oxidative stress in the body.

Oxidative stress has been suggested to be involved in the pathophysiology of many forms of hypertension;8,9 however, the exact mechanism is not yet fully understood. Scavenging of $O_2^-$ significantly reduces blood pressure in different models of hypertension,10,11 especially those associated with salt-sensitivity.12,13 Previous studies have indicated that inhibition of NO generation during high-salt intake leads to the development of salt-sensitive hypertension and the impairment of kidney function.14–16 Thus, the increases in $O_2^-$ level caused by NO deficiency may significantly contribute to the development of salt-sensitive forms of hypertension.

The specific aim of this study was to examine the role of $O_2^-$ generation in the development of salt-sensitive hypertension during chronic nitric oxide synthase (NOS) inhibition in rats. To induce NO deficiency, chronic treatment with NOS inhibitor, nitro-L-arginine methyl ester (L-NAME) was given to rats during normal and high-salt intake.14,17 Blood pressure and excretory responses were evaluated with or without co-treatment of $O_2^-$ scavenger, tempol (4-hydroxy-tetramethylpiperdine-1-oxyl), during the course of 4-week treatment with L-NAME in these rats.10,18,19

Materials and Methods

The study was performed in male Sprague-Dawley rats (Charles River Laboratories; Wilmington, Mass) in accordance with the guidelines and practices established by the Tulane University Animal Care and Use Committee. After 3 days of acclimatization, animals (220 to 250 grams body weight) were randomly divided into 6 experimental groups based on diet (normal salt, NS, 0.4% NaCl; or high-salt, HS, 4% NaCl; Harlan-Teklad, Madison, Wis) and drug treatment (NOS inhibitor, L-NAME, and $O_2^-$ scavenger, tempol;
placed in rats under anesthesia (pentobarbital, 50 mg/kg intraperitoneal). Arterial blood samples were collected from a carotid arterial cannula, and urine collections were performed in metabolic cages on the day before the start of treatment to establish basal excretory parameters and then on days 7, 14, 21, and 28 of experiment. Body weight and water intake were also recorded on each day of urine collections. The status of glomerular filtration rate (GFR) was assessed by calculating creatinine clearance to assess the changes in overall kidney function in these groups of experimental animals. We also observed that the water intake was mostly related weight gains in these animals were slightly less in L-NAME–treated animals compared with untreated group in both NS- and HS-fed rats. Such a slight decrease in weight gain was not seen in groups of rats co-treated with tempol. Thus, the retention of sodium caused by L-NAME administration during normal or high salt intake.

Analytical Methods and Statistics
Urinary excretion of sodium and potassium were assessed by flame photometry. Concentration of 8-isoprostanate in urine samples was determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, Mich).5,18 and H2O2 concentration was measured by colorimetric assay (Cayman Chemical).20 Nitrate/nitrite (NOx) concentration was also measured colorimetrically (Assay Design, Ann Arbor, Mich).6 To estimate glomerular filtration rate (GFR), creatinine clearance was calculated from the plasma and urine concentration related weight gains in these groups of experimental animals. Also, observed that the water intake was mostly remained unchanged during L-NAME or L-NAME plus tempol treatment though generally HS intake groups showed higher water intake compared with NS groups as expected (Table).

Results

Blood Pressure Responses
As shown in Figure 1, there were no significant differences in SBP at the end of 4-week experimental period in nontreated control groups with NS (127±2 to 133±2 mm Hg) or HS intake (129±2 to 136±3 mm Hg). These Sprague Dawley rats are normally not salt-sensitive as indicated by the fact that HS intake alone for 4 weeks did not significantly alter SBP. Chronic L-NAME treatment resulted in greater increases in SBP in HS rats (127±2 to 172±3 mm Hg; P<0.05) than in NS rats (130±2 to 156±2 mm Hg; P<0.05). Higher dose of L-NAME used in other studies in rats14 did not cause much difference in blood pressure responses compared with what we had observed in the present investigation indicating that this dose was sufficient to achieve a near maximal NOS inhibition. These data confirmed the previous observation14–17 that NOS inhibition leads to a salt-sensitive model of hypertension. However, co-administration of tempol with L-NAME markedly attenuated SBP in both HS (128±3 to 147±2 mm Hg; P<0.05) as well as NS rats (126±2 to 142±3 mm Hg; P<0.05).

Excretory Responses
Urine volumes collected for 24 hours are given in the Table. In addition, recorded body weight and 24-hour water intake are also given in the Table. It was observed that an age-related weight gains in these animals were slightly less in L-NAME–treated animals compared with untreated group in both NS- and HS-fed rats. Such a slight decrease in weight gain was not seen in groups of rats co-treated with tempol. Thus, the retention of sodium caused by L-NAME administration did not cause any weight gains in these groups of animals. We also observed that the water intake was mostly remained unchanged during L-NAME or L-NAME plus tempol treatment though generally HS intake groups showed higher water intake compared with NS groups as expected (Table).

The responses in urinary excretion rate of NO metabolites, nitrate/nitrite (UNOxV), to treatment with L-NAME and tempol in these experimental animals are shown in Figure 2. As expected, UNOxV was significantly lower in L-NAME–treated animals in both NS (21.1±1.2 to 14.8±0.9 μmol/d) and HS intake groups (21.8±1.6 to 11.7±0.7 μmol/d). Co-treatment with tempol did not alter the observed reduction in UNOxV induced by inhibition of NO generation either in the NS (15.6±1.3 μmol/d) or in the HS group (13.5±0.9 μmol/d).
As shown in Figure 3, urinary 8-isoprostane excretion (U ISOV) caused by chronic l-NAME treatment for 4 weeks was significantly higher in both NS (16.9±0.3 ng/d) and HS (21.5±0.8 ng/d) groups compared with corresponding NS (12.5±0.7 ng/d) and HS (13.7±0.9 ng/d) control group, indicating that NO deficiency leads to higher O₂⁻ activity. However, co-administration of tempol completely abolished responses to l-NAME in both NS (13.8±0.9 ng/d) and HS (15.8±0.9 ng/d) groups, indicating that the dose of tempol effectively reduces O₂⁻ activity in these rats during NOS inhibition. In NS rats, l-NAME treatment caused slightly but significantly lower sodium excretion on days 21 and 28 compared with the NS nontreated rats (Figure 4A). In the NS l-NAME plus tempol-treated group, sodium excretion was higher compared with the NS l-NAME–treated group. HS intake caused expected increases in sodium excretion compared with NS groups (Figure 4B). However, in the HS l-NAME group, sodium excretion was significantly lower on days 7, 14, and 21 compared with the HS nontreated group. In the HS l-NAME plus tempol-treated group, sodium excretion was significantly higher than that in the HS l-NAME group. The urinary excretion rate of H₂O₂ (UH₂O₂V) was determined in the samples collected from the experimental animals at the end of week 4 of treatment period. The results are illustrated in Figure 5. We observed that administration of l-NAME did not alter UH₂O₂V significantly in these rats fed NS or HS diets. Co-administration of tempol with l-NAME did not cause any significant increase in UH₂O₂V in both HS and NS groups. However, it was observed that UH₂O₂V was higher in nontreated HS group of rats compared with that in nontreated NS groups as reported earlier in another study. At the end of 4 weeks, creatinine clearance was calculated to determine the changes in estimated GFR (Figure 6). Attenuated creatinine clearances caused by chronic l-NAME treatment in both NS and HS rats compared with nontreated groups were partially restored in groups co-treated with tempol, suggesting that the decrease in GFR during NOS blockade is partly caused by enhancement of O₂⁻ activity in the kidney.

**Discussion**

In the present investigation, it was observed that scavenging of O₂⁻ by chronic tempol administration attenuated the SBP response to chronic l-NAME administration in rats during NS as well as HS intake. However, SBP during co-treatment of l-NAME and tempol remained significantly higher than that in the nontreated control groups (Figure 1). Urinary excretion rate of sodium was seen lower in l-NAME–treated rats but not in tempol co-treated rats in both NS and HS intake groups (Figure 4). The decreases in creatinine clearance

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**Table 1.** Body Weight, Water Intake, and Urine Volume During 4-Week Experimental Period

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW (g)</th>
<th>UV (mL/day)</th>
<th>WI (mL/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS intake</td>
<td></td>
<td>283±6</td>
<td>44.6±1.6</td>
<td>18.2±1.1</td>
</tr>
<tr>
<td>l-NAME</td>
<td>9</td>
<td>271±4</td>
<td>39.8±2.9</td>
<td>19.4±1.7</td>
</tr>
<tr>
<td>l-NAME + T</td>
<td>8</td>
<td>279±3</td>
<td>44.9±1.3</td>
<td>19.9±1.7</td>
</tr>
<tr>
<td>HS intake</td>
<td>8</td>
<td>291±4</td>
<td>46.9±1.8</td>
<td>23.8±2.4</td>
</tr>
<tr>
<td>l-NAME</td>
<td>8</td>
<td>270±6</td>
<td>40.9±2.2</td>
<td>18.8±0.9</td>
</tr>
<tr>
<td>l-NAME + T</td>
<td>8</td>
<td>283±2</td>
<td>44.8±1.9</td>
<td>18.9±1.4</td>
</tr>
</tbody>
</table>

Values are mean±SE.  
NS intake indicates normal salt; HS intake, high salt; T, Tempol; BW, body weight; UV, urine volume per day; WI, water intake per day.  
HS intake significantly increased WI and UV compared with NS intake (P<0.05).  
For the clarity of the Table, we did not include data from days 7 and 21.
(GFR) in L-NAME–treated rats were seen partially restored by co-treatment of tempol (Figure 6). Such decreases in GFR in L-NAME–treated groups were caused presumably caused by increases in pre-glomerular arteriolar resistances by the lack of NO production.\(^2\) However, partial restoration of GFR during co-treatment with tempol indicated that an enhancement of O\(_2^-\) caused by NOS inhibition also played a role in such increases in pre-glomerular resistances as suggested in earlier studies.\(^2,22\) Thus, the findings in the present study have indicated that an enhanced O\(_2^-\) activity modulates both renal hemodynamics and excretory function in the condition of NO deficiency that may be involved in the development of hypertension induced by NOS inhibition.

It has been demonstrated that NOS inhibition enhances vascular O\(_2^-\) release both in rats\(^23,24\) and in humans,\(^25\) and such enhanced O\(_2^-\) production was abolished by the use of a O\(_2^-\) scavenger.\(^25\) Although we did not measure directly the O\(_2^-\) level in the present study, we observed that UISOV (a marker for endogenous O\(_2^-\) activity) increased in L-NAME–treated rats and the response was greater in the HS group of rats compared with that in the NS group (Figure 3). These UISOV responses to L-NAME were completely prevented in rats co-treated with tempol indicating an increase in O\(_2^-\) activity during NOS inhibition. In our previous studies in dogs,\(^6,20\) we also observed an increase in UISOV during acute NOS inhibition in the kidney that was ameliorated by co-administration of tempol.

Although L-NAME–induced SBP response was much greater in the HS intake group compared with the NS intake group, tempol treatment caused attenuation of SBP to similar levels in both groups that are not significantly different from each other (Figure 1). Because tempol administration abolished the differences in hypertensive responses to L-NAME during varying salt intake, these findings indicate that the development of salt-sensitivity induced by chronic NOS inhibition is mainly attributed to increases in endogenous O\(_2^-\) activity. Tempol treatment did not alter UISOV in the NOS inhibited rats, indicating that the blood pressure lowering effect of tempol in the present study was not caused by reversal of NO bioavailability but rather was caused by decreases in O\(_2^-\) activity. It has also been shown that tempol treatment significantly attenuates blood pressure in several hypertensive models that are particularly associated with salt-sensitivity.\(^12,13,18,26\) Thus, the results of present investigation further support an important role of O\(_2^-\) in the development of salt-sensitivity in NO-deficient hypertension.

It may be argued that tempol as a SOD mimetic lead to increases in H\(_2\)O\(_2\) levels that influence the SBP responses in rats in the present investigation. However, it was observed that tempol administration in L-NAME–treated rats did not cause any significant increase in UH\(_2\)O\(_2\)V in both HS and NS groups (Figure 5). Thus, it would argue against any significant contribution of H\(_2\)O\(_2\) in the observed marked attenuation of the SBP responses to tempol in L-NAME–treated rats. Although the effects of catalase administration after tempol treatment had not been examined in the present study, we have reported earlier that acute administration of tempol did not alter UH\(_2\)O\(_2\)V either in dogs\(^20\) or in normotensive, as well

![Figure 3. Urinary 8-isoprostane excretion responses to chronic L-NAME and Tempol (T) treatment during normal or high salt intake. *P < 0.05 vs nontreated group; #P < 0.05 vs L-NAME group.](attachment:image)

![Figure 4. Urinary sodium excretion responses to chronic L-NAME and Tempol (T) treatment during normal or high salt intake. *P < 0.05 vs nontreated group; #P < 0.05 vs L-NAME group.](attachment:image)
as angiotensin II–induced hypertensive rats. We also observed that there was no significant difference between the renal responses to intra-arterial administration of tempol with or without catalase in rats. As reported previously, UH_{2O2V} was seen higher in nontreated HS group of rats compared with that in nontreated NS groups in our present study. It is to be noted here that such differences in UH_{2O2V} was observed, although blood pressure levels are similar in both nontreated NS and HS groups. However, the effects of a possible change in vascular level of H_{2O2} during tempol administration are yet to be determined conclusively. It has been shown that H_{2O2} acts as a vasodilator, implicating that enhancement of its vascular level would cause a decrease in blood pressure. However, some studies have implicated that increases in renal medullary tissue H_{2O2} level causes hypertension in rats, an effect that is opposite to the present finding of tempol-induced reduction in SBP.

Oxidative stress and hypertension are closely associated with higher sympathetic activity. Thus, it could be argued that tempol-induced changes in blood pressure observed in the present study may be influenced by its inhibitory effects on sympathetic activity or antioxidant-induced changes in the release of norepinephrine from the nerve terminals. However, it was also demonstrated that enhanced O_{2}^{–} activity by SOD inhibition can cause stimulation of sympathetic activity that was shown to be inhibited by tempol. Thus, reduction of endogenous O_{2}^{–} activity by tempol administration can indirectly be associated with a possible reduction in sympathetic activity in hypertensive rats in the present study.

The exact mechanism that produces increases in the endogenous level of O_{2}^{–} during NOS inhibition is not yet clear. Both NO and O_{2}^{–} are constant products of cellular metabolism, and both of these molecules are constantly interacting with each other in biological tissues. Normally, O_{2}^{–} in the tissue is kept to a minimal level by the antioxidative function of SOD as well as NO. However, when NO production is diminished in the tissue, it is expected that this balance may be altered allowing O_{2}^{–} accumulation in the tissue because of its inadequate removal by NO. It is also possible that the activity of enzymes responsible for endogenous production of O_{2}^{–} may be upregulated during NOS inhibition. Further experiments are required to determine the activity of these oxidative enzymes during NO synthase inhibition.

In conclusion, these data demonstrate that the enhanced O_{2}^{–} activity caused by chronic NOS inhibition contributes to the development of salt sensitivity that is involved in the pathophysiology of the NO-deficient form of hypertension.

**Perspectives**

The findings of this present study further support our previous observations indicating an important role of the interaction between O_{2}^{–} and NO in the regulation of renal function and blood pressure. NO provides a protective role against the actions of O_{2}^{–} by acting as an important antioxidative agent in the body. The development of any imbalance between oxidative and antioxidative processes in living tissues would lead to derangements in organ function including the kidney. The results of the present study, which demonstrate a close relation between enhancement of O_{2}^{–} activity and the development of salt sensitivity during NOS inhibition, provide an important clue in our quest in understanding the pathophysiology of salt-sensitive hypertension. Thus, it is imperative that further emphasis should be focused on complete elucidation of the interactive role of O_{2}^{–} and NO in the regulation of many organ functions to increase our knowledge on physiology as well as pathophysiological processes of many diseases that are linked to NO metabolism and oxidative stress.

**Acknowledgments**

We gratefully acknowledge the technical help provided by Alexander Castillo and Kevin Wellen. This study was supported by National Heart, Lung, and Blood Institute grant HL-51306.

**References**


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Hypertension. 2005;46:1026-1031; originally published online August 15, 2005; doi: 10.1161/01.HYP.0000174989.39003.58

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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